EXHIBIT 1

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Hepatitis C Virus Glycoprotein Folding: Disulfide Bond Formation and Association with Calnexin

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The hepatitis C virus (HCV) glycoproteins (E1 and E2) are released from the polyprotein by signal peptidase-mediated cleavage and interact to form a heterodimer. Since properly folded subunits are usually required for specific recognition and stable oligomer formation, the rate of stable E1E2 complex formation, which is low, may be limited by the rate of HCV E1 and/or E2 folding. In this study, the folding of the HCV E1 and E2 glycoproteins was monitored by observing the kinetics of intramolecular disulfide bond formation. The association/dissociation of E1 and E2 with calnexin was also examined, since this molecular chaperone appears to play a major role in quality control via retention of incompletely folded or misfolded proteins in the endoplasmic reticulum. Our results indicate that the disulfide-dependent folding of E2 occurs rapidly and appears to be complete upon cleavage of the precursor E2-NS2. In contrast, folding of E1 is slow (>1 h), suggesting that this step may be rate limiting for E1E2 oligomerization. Both HCV glycoproteins associated rapidly with calnexin, but dissociation was slow, consistent with the slow folding and assembly of E1E2 glycoprotein complexes. These results suggest a role for prolonged association with calnexin in the folding and assembly of HCV glycoprotein heterodimer complexes.

Hepatitis C virus (HCV), the major causative agent of non-A, non-B hepatitis (10, 40), is an enveloped virus containing a positive-strand RNA genome of approximately 9,500 nucleotides (10, 11). HCV (36, 47) has a similar genomic organization to the pestiviruses (12) and the flaviviruses (8), and these groups have recently been classified as three genera in the family *Flaviviridae* (21). The HCV genome contains a highly conserved 5′ noncoding region (28) followed by a long open reading frame of 9,030 to 9,099 nucleotides that is translated into a single polyprotein of 3,010 to 3,033 amino acids (47). This polyprotein is processed by a combination of host and viral proteinases to produce the putative viral structural and nonstructural (NS) proteins (2, 24, 25, 32–34, 44, 56, 59). The order and nomenclature of the cleavage products in the polyprotein is NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (26, 43).

The HCV nucleocapsid protein (C) is followed by two putative virion envelope glycoproteins, E1 and E2, which contain 5 or 6 and 11 N-linked glycosylation sites, respectively (48). Previous studies indicate that a host signal peptidase localized in the endoplasmic reticulum (ER) catalyzes polyprotein cleavages in the structural region (C/E1, E1/E2, E2/p7, and p7/NS2) (32, 43), whereas an HCV-encoded serine proteinase located in the N-terminal one-third of the NS3 protein, is responsible for four cleavages in the NS region (3/4A, 4A/4B, 4B/5A, and 5A/5B) (2, 20, 24, 33, 44, 59). Cleavage at the 2/3 site is mediated by a second HCV-encoded proteinase, which encompasses the NS2 region and the NS3 serine proteinase (25, 33). Cleavages at the C/E1, E1/E2, and NS2/NS3 sites are cotranslational, whereas those at the E2/p7 and p7/NS2 sites occur posttranslationally and generate two precursors for E2: E2-NS2 and E2-p7 (26, 43, 49, 57). Although E2-p7 is relatively stable for the HCV-1, HCV-1b_J, and H strains, other studies with a construct derived from a distinct HCV subtype, HCV-BK (58), suggest that processing at the E2/p7 site might be more efficient (19, 43).

The low levels of HCV particles present in patient plasma and the lack of an efficient cell culture system for HCV propagation have precluded a direct analysis of the virion envelope glycoproteins. Previous reports suggest that E1 and E2 interact to form a complex, which has been proposed as a functional subunit of HCV virions (19, 26, 42, 55). Purified HCV glycoprotein complexes expressed by using vaccinia virus are noncovalently associated (55). In contrast, a fraction of E1 and E2 present in lysates of cells infected with vaccinia virus-HCV recombinants has been reported to be associated via disulfide linkages (26). Recently, we have characterized the complexes formed between E1 and E2 (19). In the presence of nonionic detergents, two forms of E1E2 complexes are detected: a heterodimer of E1 and E2, which is stabilized by noncovalent interactions, and heterogeneous disulfide-linked aggregates, which most probably represent misfolded complexes. The kinetics of association between E1 and E2 indicate that formation of stable E1E2 complexes is slow. The time required for half-maximal association between E1 and E2 is 60 to 85 min for the H strain and more than 165 min for the BK strain. Since properly folded subunits are usually required for specific recognition and stable association, these results suggest that the rate of HCV E1 and E2 folding, which probably depends on interactions with molecular chaperones in the ER, may limit the rate of complex formation.

The efficacy of folding depends on the partitioning of an unfolded protein between productive pathways leading to the native structure and nonproductive pathways leading to aggregation and misfolding (50). In vivo folding and assembly of newly synthesized proteins often involve a variety of cellular folding enzymes and molecular chaperones (23). Cellular foldases increase the rate and yield of productive folding by catalyzing slow chemical steps, such as disulfide bond forma-

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tion and proline isomerization, that accompany the correct folding and maturation of proteins. On the other hand, molecular chaperones bind to unfolded proteins and prevent nonproductive folding and aggregation (30). In addition to the major soluble chaperones of the ER lumen (23, 30), recent work from several laboratories has identified a new type of chaperone that is an integral component of the ER membrane (3). This chaperone, called calnexin, associates transiently with numerous newly synthesized polypeptides and folding intermediates of secretory glycoproteins during their maturation. It has been hypothesized that calnexin may function as a molecular chaperone that retains incompletely assembled oligomers in the ER (3).

In this study, the folding of the HCV E1 and E2 glycoproteins was monitored by observing the kinetics of intramolecular disulfide bond formation. We also examined the association-dissociation of E1, E2, and E1E2 oligomer with calnexin. Our results indicate that the disulfide-dependent folding of E1 is slow, which may limit the rate of E1E2 oligomer formation. In addition, both HCV glycoproteins associated rapidly with calnexin but dissociated slowly. These results are consistent with the slow folding and assembly of E1E2 glycoprotein complexes.

MATERIALS AND METHODS

Cells and viruses. The CV-1 and HepG2 cell lines were obtained from the American Type Culture Collection, Rockville, Md. Cell monolayers were grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum.

Stocks of vTF7-3, a vaccinia virus recombinant expressing the T7 DNA-dependent RNA polymerase (22), and vaccinia virus-HCV (vHCV1-1488), which contains sequences of the HCV-H strain (26), were grown in CV-1 monolayers and partially purified (37), and titers of infectious progeny were determined by plaque assay on CV-1 cells.

HCV-specific antisera and MAbs. Anti-E1 (A4) and anti-E2 (A11) monoclonal antibodies (MAbs) and anti-NS2 antiserum have been described previously (19, 26). MAb AF8 and antiserum to calnexin were kindly provided by M. B. Brenner and J. M. Bergeron, respectively (35, 51). Anti-BVDV MAb NY-8 has been described previously (4).

Metabolic labeling and immunoprecipitation. Subconfluent monolayers in 35-mm dishes were infected with the appropriate recombinant virus at a multiplicity of infection of 5 to 10 PFU per cell. After 1 h at room temperature, medium containing 5% fetal bovine serum was added. Between 4 and 4.5 h postinfection, monolayers were washed once with prewarmed medium lacking methionine and cysteine and incubated in the same medium for an additional 30 min. Infected cells were then pulse-labeled for 5 min with 100 μCi of [^{35}S]translabel (ICN) per ml. Cells were washed twice with prewarmed medium containing a 10-fold excess of methionine and cysteine, followed by a chase for various times in this medium. Cells were then lysed with 0.5% Nonidet P-40 (NP-40) in 10 mM Tris-Cl (pH 7.5)–150 mM NaCl–2 mM EDTA. Iodoacetamide (20 mM) was included in the lysis buffer for experiments in which disulfide bond formation was determined. Cell lysates were clarified by centrifugation in an Eppendorf centrifuge for 15 min. For steady-state labeling, cells were labeled at 4 h postinfection with 25 μCi of [^{35}S]translabel (2 h of labeling) per ml in medium containing 1/40 the normal concentration of methionine and 2% fetal bovine serum. For in vivo reduction (5), the culture medium was replaced by medium containing 5 mM dithiothreitol (DTT).

Immunoprecipitations of nondenatured antigen were carried out as described previously (29) with some modifications. A 6-μl volume of rabbit anti-mouse immunoglobulin G (DAKO) was incubated with protein A-Sepharose (Pharmacia-LKB) for 1 h at 4°C in a solution containing 0.2% NP-40, 10 mM Tris-Cl (pH 7.5), 150 mM NaCl, and 2 mM EDTA (TBS–NP-40). This step was omitted when anti-NS2 or anti-calnexin antiserum was used. Beads were then incubated with 1 μl of MAb or 2 μl of a rabbit antiserum, followed by the antigen (each step for 1 h at 4°C). Between each step, the beads were washed once with TBS–NP-40. After the last step, they were washed three times with this buffer and once with distilled water. NP-40 was replaced by digitonin for immunoprecipitations with anti-calnexin antibodies. The precipitates were then boiled for 5 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and run on a 10 or 12% polyacrylamide gel (41). After electrophoresis, the gels were treated with sodium salicylate (7), dried, and exposed at −70°C to preflashed Hyperfilm-MP (Amersham). The intensity of the bands was determined by densitometry of autoradiograms. ^{14}C-methylated protein molecular mass markers were purchased from GIBCO-BRL.

Sedimentation through sucrose gradients. Labeled HCV glycoproteins bound to calnexin were immunoprecipitated with an anti-calnexin MAb and dissociated by incubation for 5 min at 37°C in lysis buffer containing 0.5% NP-40. The supernatant was layered on a 10-ml gradient of 5 to 20% sucrose in TBS containing 0.1% NP-40. After centrifugation at 4°C for 24 h at 36,000 rpm in a Beckman SW41 rotor, 11 fractions were collected from the bottom of the gradient and analyzed by immunoprecipitation as described above. Labeled infected cells lysed with 0.5% NP-40 were used as a control. Molecular mass markers (Combithek, calibration protein I; Boehringer-Mannheim) were sedimented in a parallel sucrose gradient.

Enzyme digestions. The immunoprecipitated proteins were eluted from protein A-Sepharose in 30 μl of 0.5% SDS by boiling for 10 min and were digested with PNGase F (New England Biolabs). Digestions with PNGase F were carried out for 1 h at 37°C in the buffer provided by the manufacturer. Digested samples were mixed with an equal volume of 2× Laemmli sample buffer with or without 20 mM DTT and 2% β-mercaptoethanol and analyzed by SDS-PAGE.

Western blotting (immunoblotting). Proteins bound to nitrocellulose membranes (Hybond-ECL; Amersham) were analyzed by enhanced chemiluminescence detection (ECL; Amersham) as recommended by the manufacturer. Briefly, after separation by SDS-PAGE, proteins were transferred to nitrocellulose membranes by using a Trans-Blot apparatus (Bio-Rad) and revealed with specific MAbs (dilution of 1/2,000) followed by goat anti-mouse immunoglobulin conjugated to horseradish peroxidase (dilution of 1/1,000; DAKO).

RESULTS

Disulfide bond formation and folding of the HCV glycoproteins. SDS-PAGE under nonreducing conditions has been used to monitor disulfide bond formation in viral glycoproteins (17). This method takes advantage of an increase in mobility as a protein acquires more compact conformations stabilized by the formation of disulfide bonds and often reflects progressive folding toward the native state. With 8 cysteine residues for E1 and 20 for E2 (13), the HCV glycoproteins are expected to become disulfide bridged. This was tested by conducting pulse-chase experiments with a vaccinia virus-HCV recombinant to express the HCV glycoproteins in HepG2 cells. Cells were lysed in the presence of iodoacetamide to prevent further disulfide bond formation, and the viral proteins were precipitated with anti-E1 or -E2 MAb. Precipitates were split into two equal portions, one portion was treated with DTT and β-mercaptoethanol, and both portions were analyzed by SDS-PAGE.

The disulfide-dependent folding of E1 is slow. Immediately following the pulse and up to 15 min of chase, a single E1 species was detected under both reducing and nonreducing conditions (Fig. 1). When these samples were analyzed on the same gel, nonreduced and reduced E1 comigrated (data not shown), suggesting that intramolecular disulfide bonds had not yet formed during the pulse-label or the first 15 min of chase. The electrophoretic mobility of E1 increased after longer periods of chase. After 30 or 60 min, E1 appeared more heterogeneous, which presumably results from different conformations stabilized by disulfide bonds. After 60 min, but seen more clearly after 120 min, a second form of E1 (E1ox) with a greater electrophoretic mobility was readily detected. We suggest that this form represents a more compact conformation resulting from formation and/or reshuffling of disulfide bonds. The intensity of the oxidized form of E1 increased slowly during the chase, suggesting that the formation and/or reshuffling of disulfide bonds in E1 is a slow process. As seen most clearly for reduced samples, the mobility of E1 increased progressively during the chase, presumably as a result of trimming of mannose-rich oligosaccharide chains. Other species detected included coprecipitated E2, E2-NS2, and, for nonreduced samples, disulfide-linked complexes involving E1 and E2.

Disulfide-dependent folding of E2-NS2 and E2. As shown in earlier studies (26, 43), processing in the E2-NS2 region is complex. A discrete E2-NS2 precursor which is processed to produce E2 and E2-p7 can be identified (Fig. 2). These glycosylated products are heterogeneous and poorly resolved but

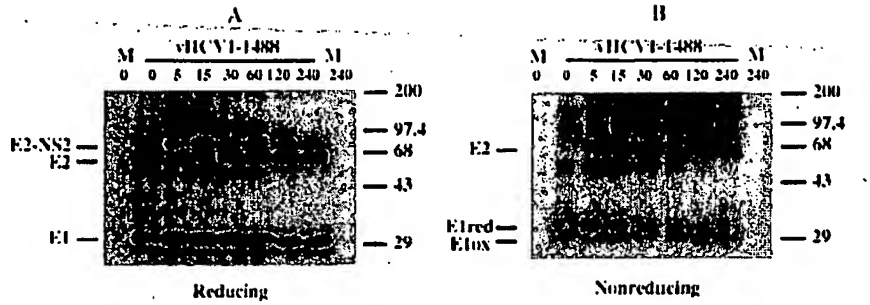

FIG. 1. Oxidation of the E1 glycoprotein. Cells coinfected with vTF7-3 and vHCV1-1488 were pulse-labeled for 5 min and chased for the indicated times (in minutes). The E1 glycoprotein was immunoprecipitated with anti-E1 MAb. Immunoprecipitates were analyzed under reducing (A) and nonreducing (B) conditions by SDS-PAGE (10% polyacrylamide). vTF7-3 alone was used as a control (lanes M). HCV-specific proteins are indicated at the left, and the sizes (in kilodaltons) of protein molecular mass markers are indicated at the right.

can be visualized clearly after deglycosylation (26, 43) (Fig. 2C and D). As shown in Fig. 2A, E2-related products were heterogeneous after a 5-min pulse-label, yielding a prominent 70- to 75-kDa species migrating more slowly than mature E2 and E2-p7. After deglycosylation, this product appeared to migrate at 42 kDa, which is consistent with an E2-related species including the p7 region and the N-terminal part of NS2 (Fig. 2C). This species does not appear to be E1 related (Fig. 1) or to be a degradation product, since after a 5-min chase, the majority of the label can be found in E2-NS2 and minor amounts of E2 and E2-p7 cleavage products (Fig. 2A and C). Although further characterization is needed, this species may result from a translational pause during synthesis of the NS2 region. In any case, the E2 and E2-p7 cleavage products were apparent after only a 5-min chase and the electrophoretic mobilities of their deglycosylated forms under nonreducing conditions did not increase markedly with extended periods of chase (Fig. 2D). Other E2-related species, including E2-NS2 and disulfide-linked complexes involving E1 and E2, were also detected in the upper part of nonreducing gels (Fig. 2B and D). As shown most clearly in Fig. 2E, the nonreduced forms of E2 and E2-p7 migrated faster than did their reduced counterparts, suggesting that they had already acquired a compactly folded conformation. Taken together, these results suggest that disulfide bond formation and/or reshuffling for E2 is rapid and occurs before or shortly after proteolytic processing of E2-NS2.

To examine disulfide bond formation in the E2-NS2 precursor, we used an NS2-specific antiserum for immunoprecipitation to eliminate disulfide-linked E1E2 complexes of similar mobility (Fig. 3). Nonreduced E2-NS2 did not migrate faster than its reduced form, and an increase in mobility could not be observed during the chase (Fig. 3A and B). However, after

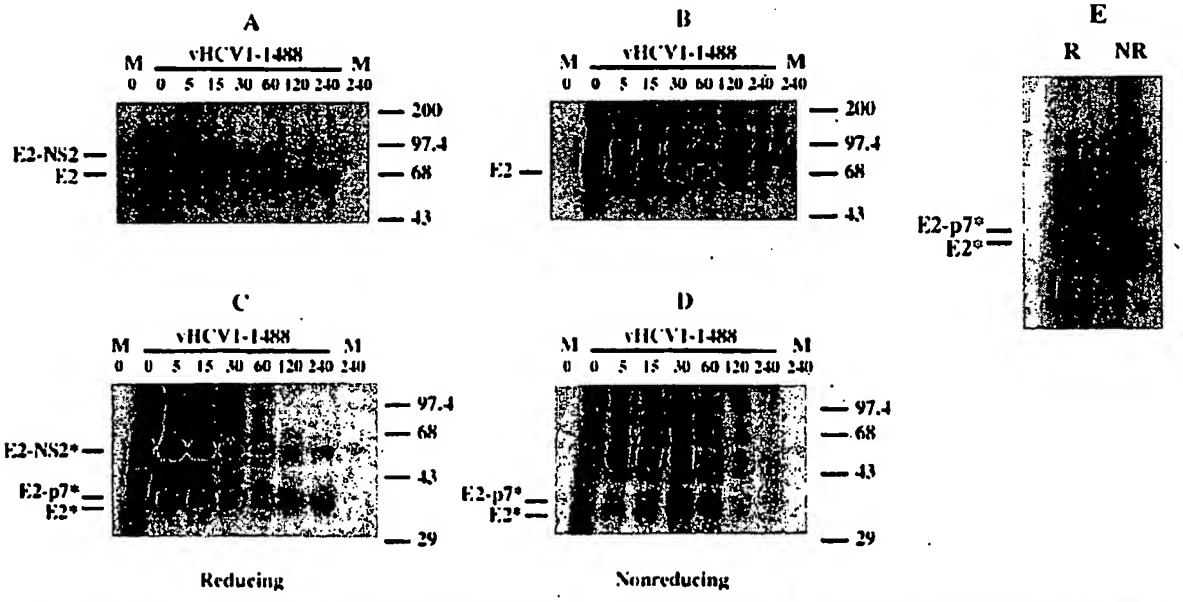

FIG. 2. Analysis of disulfide bond formation for the E2 glycoprotein. Cells coinfected with vTF7-3 and vHCV1-1488 were pulse-labeled for 5 min and chased for the indicated times (in minutes). The E2 glycoprotein was immunoprecipitated with anti-E2 MAb. Immunoprecipitates were analyzed under reducing (A, C, and E [lane R]) or nonreducing (B, D, and E [lane NR]) conditions by SDS-PAGE (10% polyacrylamide). To remove N-linked glycans, HCV glycoproteins were treated with PNGase F (C to E) before being separated by SDS-PAGE. vTF7-3 alone was used as a control (lanes M). HCV-specific proteins are indicated at the left (deglycosylated proteins are indicated by asterisks), and the sizes (in kilodaltons) of protein molecular mass markers are indicated at the right.

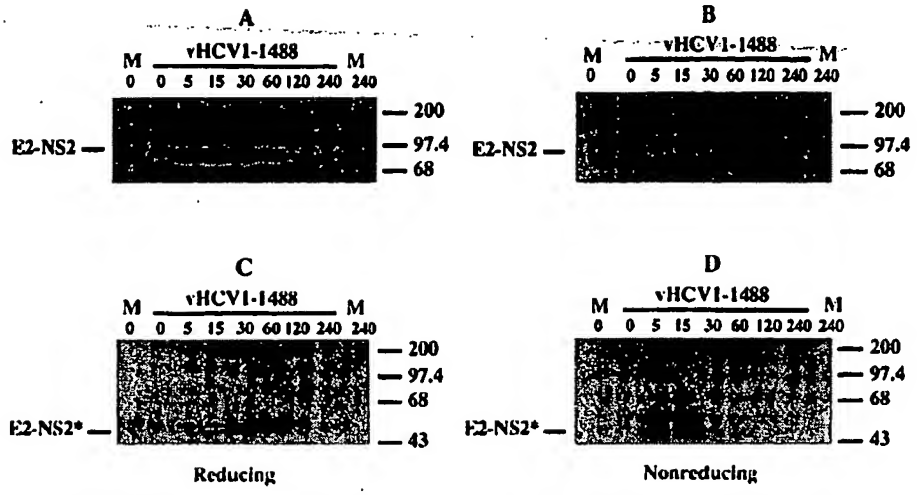

FIG. 3. Analysis of E2-NS2 glycoprotein under reducing and nonreducing conditions. Cells coinfected with vTF7-3 and vHCV1-1488 were pulse-labeled for 5 min and chased for the indicated times (in minutes). The E2-NS2 glycoprotein was immunoprecipitated with an anti-NS2 antiserum. Immunoprecipitates were analyzed under reducing (A and C) or nonreducing (B and D) conditions by SDS-PAGE (10% polyacrylamide). N-linked glycans were removed by treatment of some samples with PNGase F (C and D). vTF7-3 alone was used as a control (lanes M). HCV-specific proteins are indicated at the left (deglycosylated proteins are indicated by asterisks), and the sizes (in kilodaltons) of protein molecular mass markers are indicated at the right.

deglycosylation, the nonreduced forms of E2-NS2 were initially heterogeneous but resolved into two predominant species (Fig. 3D). An increased proportion of the faster-migrating E2-NS2 form was apparent after longer chases, which indicates that the E2-NS2 precursor had assumed a more compact conformation stabilized by intramolecular disulfide bonds.

These results indicate that formation of intramolecular E2 disulfide bonds occurs prior to E2-NS2 processing, raising the possibility that this conformation of the E2 region is required for further proteolytic processing. To test this hypothesis, disulfide bonds were reduced in vivo by addition of DTT to the medium of infected cells (5). Under nonreducing electrophoresis conditions, E2-NS2 from DTT-treated cells migrated as a reduced protein, confirming the reducing effect of DTT in vivo (data not shown). As shown in Fig. 4, the kinetics of E2-NS2 cleavage were similar in the presence and absence of DTT, suggesting that E2-NS2 cleavage is not dependent on a change in E2 conformation stabilized by disulfide bonds.

HCV glycoproteins coprecipitate with calnexin. Since calnexin is believed to function as a molecular chaperone which retains incompletely assembled glycoproteins in the ER (3), we examined the association of HCV glycoproteins with calnexin. Digitonin, which does not disrupt calnexin-glycoprotein complexes, was used to prepare lysates for coprecipitation experiments. With an anti-calnexin MAb, three radiolabeled species corresponding in size to HCV E2-NS2, E2, and E1 were detected (Fig. 5A). The same bands were coprecipitated with a calnexin-specific antiserum (data not shown). These proteins

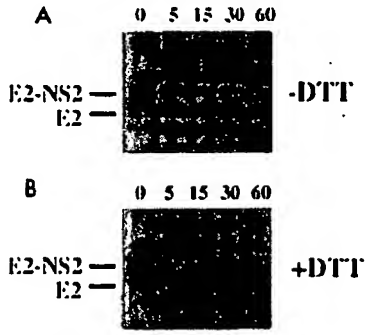

FIG. 4. Effect of in vivo reduction on E2-NS2 cleavage. Cells infected with vTF7-3 plus vHCV1-1488 were pulse-labeled for 5 min and chased for the indicated times (in minutes). Infected cells were not treated (A) or treated (B) with 5 mM DTT during the pulse and chase periods. E2 and E2-NS2 glycoproteins were immunoprecipitated with anti-E2 MAb. Immunoprecipitates were analyzed under reducing conditions by SDS-PAGE (10% polyacrylamide). HCV-specific proteins are indicated at the left.

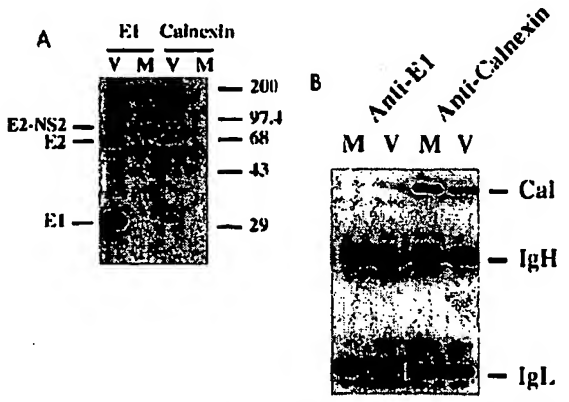

FIG. 5. Coprecipitation of HCV glycoproteins with calnexin. Cells coinfected with vTF7-3 and vHCV1-1488 (lanes V) or vTF7-3 alone (lanes M) were labeled with [^{35}S]methionine and lysed with 1% digitonin. Cell lysates were clarified and used for immunoprecipitation with anti-E1 or anti-calnexin MAbs. Immunoprecipitates were revealed by autoradiography (A) or Western blotting with anti-calnexin MAb (B). HCV-specific proteins are indicated at the left, and the sizes (in kilodaltons) of protein molecular mass markers are indicated at the right. Cal, calnexin; IgH and IgL, immunoglobulin heavy and light chains, respectively.

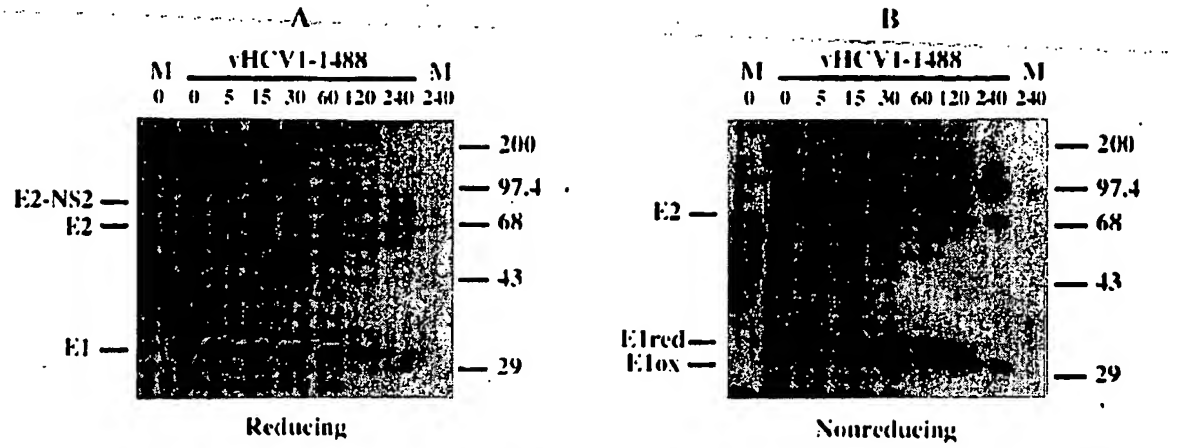

FIG. 6. Rate of HCV glycoprotein association with calnexin. Cells coinfected with vTF7-3 and vHCV1-1488 were pulse-labeled for 5 min and chased for the indicated times (in minutes). Labeled infected cells were lysed with 1% digitonin and used for immunoprecipitation with anti-calnexin MAb. Immunoprecipitates were analyzed under reducing (A) or nonreducing (B) conditions by SDS-PAGE (10% polyacrylamide). vTF7-3 alone was used as a control (lanes M). HCV-specific proteins are indicated at the left, and the sizes (in kilodaltons) of protein molecular mass markers are indicated at the right.

were not precipitated with an isotype-matched control MAb, and their identities were confirmed by Western blotting with anti-E1 and anti-E2 HCV MAbs (data not shown). Radiolabeled calnexin (which migrates at 90 kDa) was not readily detected under these conditions, perhaps because of poor metabolic labeling in vaccinia virus-infected cells. However, interaction with calnexin was confirmed in a reciprocal experiment, in which 90-kDa calnexin was readily detected by Western blotting of lysates immunoprecipitated with anti-E1 MAb (Fig. 5B). The results suggest that the HCV glycoproteins interact with ER-resident calnexin, either directly or indirectly, through association with each other or as yet unidentified host components.

Kinetics of association with calnexin. To examine the rate of HCV glycoprotein interaction with calnexin, a pulse-chase experiment was performed and digitonin-solubilized lysates were immunoprecipitated with anti-calnexin MAb (Fig. 6). After a 5-min pulse-label, the majority of HCV E1 coprecipitated with anti-calnexin MAb (Fig. 6A) (see below). Similar coprecipitation was observed for E2-NS2. Overall, the pattern of HCV glycoproteins and glycoprotein precursors that coprecipitated with calnexin was very similar to the results obtained above with HCV-specific MAbs. This suggests that association of HCV glycoproteins with calnexin is rapid and their dissociation is slow. A more careful comparison is shown in Fig. 7. In this experiment, the level of each HCV glycoprotein coprecipitated with calnexin MAb was compared with the level immunoprecipitated with HCV glycoprotein-specific MAbs. Maximal coprecipitation of E2-NS2 with calnexin MAb was observed after a 15-min chase, and its disappearance from coprecipitates followed the rate of E2-NS2 cleavage. Concomitant with the disappearance of E2-NS2, the level of E2 (or E2-p7) increased with maximal coprecipitation by calnexin MAb after 60 min of chase. Coprecipitation of E1 peaked after a short chase and declined throughout the chase period.

Interaction with calnexin and formation of intramolecular disulfide bonds. As mentioned above, formation and/or redistribution of disulfide bonds can be accompanied by changes in protein conformation. For transferrin, the acquisition of disulfide bonds (conversion to the oxidized form) has been correlated with its dissociation from calnexin (51). This was examined for HCV E1 by determining which forms were associated with calnexin during the conversion of E1red (lower-mobility form) to E1ox (higher-mobility form). As shown in Fig. 6B, the oxidized form of E1 could be coprecipitated with calnexin, indicating that disulfide maturation of E1 does not induce complete dissociation from calnexin. However, it was apparent that the level of E1ox coprecipitating with calnexin decreased dramatically during the chase. The association of E1ox with calnexin may indicate that another step in glycoprotein folding and/or assembly is necessary for complete dissociation from calnexin.

Evidence that E1 and E2 can interact independently with calnexin. Since the HCV glycoproteins interact to form E1E2 complexes (19), coprecipitation of E1 or E2 with anti-calnexin MAb could reflect an interaction with either or both HCV glycoproteins (or an unlabeled host component). The ability of each HCV glycoprotein to interact with calnexin was examined by dissociating anti-calnexin immune complexes by heating them for 5 min at 37°C in the presence of 0.5% NP-40 and reprecipitating the supernatants with anti-E1 or anti-E2 MAbs. After a 5-min chase, the major species precipitated by anti-E1 MAb was HCV E1 and the major species precipitated by anti-E2 MAb were E2-NS2 and E2 (Fig. 8). These results suggest that each of the HCV glycoproteins, at least early after synthesis, can interact with calnexin. Nevertheless, a small amount of E2-NS2 was coprecipitated with the anti-E1 MAb and a faint band corresponding to E1 was coprecipitated with the anti-E2 MAb, suggesting that some complexes were also associated with calnexin. This was clearer after a 120-min chase, when the majority of the HCV glycoproteins that coprecipitated with anti-calnexin MAb were E1E2 complexes (Fig. 8) (see below).

Interaction of calnexin with E1E2 heterodimers. The formation and size of HCV glycoprotein complexes associated with calnexin were examined in a pulse-chase analysis by dissociating the anti-calnexin immune complexes with NP-40 followed by sedimenting through sucrose gradients containing the same detergent (Fig. 9, left). For comparison, HCV glycoproteins present in total NP-40-solubilized extracts were run in parallel gradients (Fig. 9, right). The association of E2 with E1 was monitored by coprecipitation of E2-specific products with an-

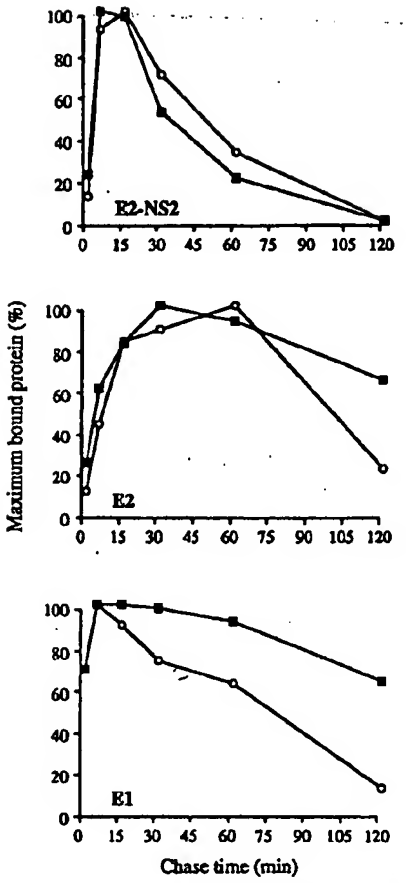

FIG. 7. Kinetics of E1, E2, and E2-NS2 association with calnexin. Cells coinfected with vTF7-3 and vHCV1-1488 were pulse-labeled for 5 min and chased for the indicated times (in minutes). Labeled infected cells were lysed and used for immunoprecipitation with either anti-calnexin, anti-E1, or anti-E2 MAbs. Immunoprecipitates were analyzed by SDS-PAGE (10% polyacrylamide), and quantitation was performed by scanning densitometry of fluorographs. The amount of viral protein precipitated is expressed as a percentage of the maximal amount coprecipitated with calnexin MAb (○), or anti-E1 or -E2 MAb (■).

DISCUSSION

Our results suggest a low rate for disulfide-dependent folding of the HCV E1 glycoprotein. In contrast, both E2 and E2-p7 appear to be present in a compact configuration prior to or immediately after proteolytic processing of E2-NS2. Since properly folded subunits are usually required for specific recognition and stable association, these data suggest that correct folding of E1 could be rate limiting for formation of E1E2 oligomers. Shortly after synthesis, both E1 and E2-NS2 were found to be associated with the ER chaperone calnexin. Calnexin-associated E1 was initially present as a monomer, which was subsequently converted to E1E2-NS2 and then to E1E2 complexes. Most of these complexes appeared to be heterodimers on the basis of their sedimentation behavior on sucrose gradients. Although assembly of quaternary complexes of mature glycoproteins often signals their release from calnexin and continued transit through the secretory pathway, we found that a significant fraction of E1E2 oligomers remained associated with calnexin. These results suggest a role for prolonged association with calnexin in the folding and assembly of HCV glycoprotein heterodimer complexes.

Although these data might suggest an orderly stepwise pathway for the folding, disulfide rearrangement, and assembly of HCV glycoprotein oligomers, other observations indicate that the situation is more complicated. A significant fraction of newly synthesized E1 forms heterogeneous complexes with E2-related species which are stabilized by disulfide bonds. The slow folding and oxidation of E1 may encourage the formation of E1E2 complexes linked by intermolecular disulfide bonds. Such complexes are still present even after 4 h of chase and, as we previously suggested (19), may represent misfolded dead-end complexes. Alternatively, disulfide rearrangement in these complexes may lead to the formation of correctly assembled functional oligomers, either noncovalently associated or containing intermolecular disulfide bonds. Although it is commonly believed that noncovalently associated E1E2 heterodimers are the functional subunits of the HCV virion envelope, the actual oligomeric structure of HCV glycoproteins on infectious virions has not been examined.

In contrast to E1, disulfide-dependent folding of E2 and E2-p7 appeared to be complete by the time of E2-NS2 cleavage. This observation indicates that disulfide-dependent folding of these domains occurs prior to or soon after cleavage. A possible explanation for the delayed processing of E2-NS2 is a requirement for disulfide-dependent rearrangement to pro-

ti-E1 MAb. After a 5-min chase, E1, sedimenting more slowly than the 68-kDa marker, was the major calnexin-associated species. In contrast, total E1 extracted from cells was distributed more widely in the gradient and E2-NS2 was detected in some fractions. These results suggest that monomeric E1 is the major calnexin-associated form near the beginning of the chase and that some complexes (E1E2-NS2 heterodimers and perhaps E1 multimers) which are not associated with calnexin are present. After a 45-min chase, significant amounts of E2-NS2 and E2 coprecipitated with E1, and these calnexin-associated complexes sedimented between 68- and 158-kDa mass standards. At this time, a significant peak of E1 sedimenting more slowly than the 68-kDa marker was still apparent. After a 120-min chase and conversion of E2-NS2 to E2, the major calnexin-associated species appeared to be E1E2 heterodimers sedimenting between the 68- and 158-kDa markers. Similar results were obtained for the glycoproteins present in total NP-40 extracts.

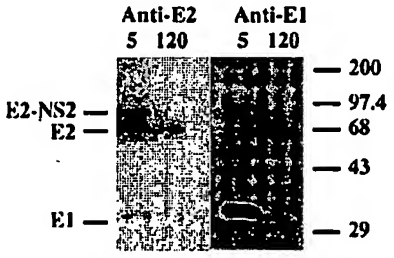

FIG. 8. Both HCV glycoproteins can interact with calnexin. Cells coinfected with vTF7-3 and vHCV1-1488 were pulse-labeled for 5 min and chased for the indicated times (in minutes). Labeled infected cells were lysed with 1% digitonin and used for immunoprecipitation with anti-calnexin MAb. Coprecipitated HCV glycoproteins were dissociated from calnexin by heating for 5 min at 37°C in 0.5% NP-40 and reprecipitated with anti-E1 or -E2 MAbs. Immunoprecipitates were analyzed under reducing conditions by SDS-PAGE (10% polyacrylamide). HCV-specific proteins are indicated at the left, and the sizes (in kilodaltons) of protein molecular mass markers are indicated at the right.

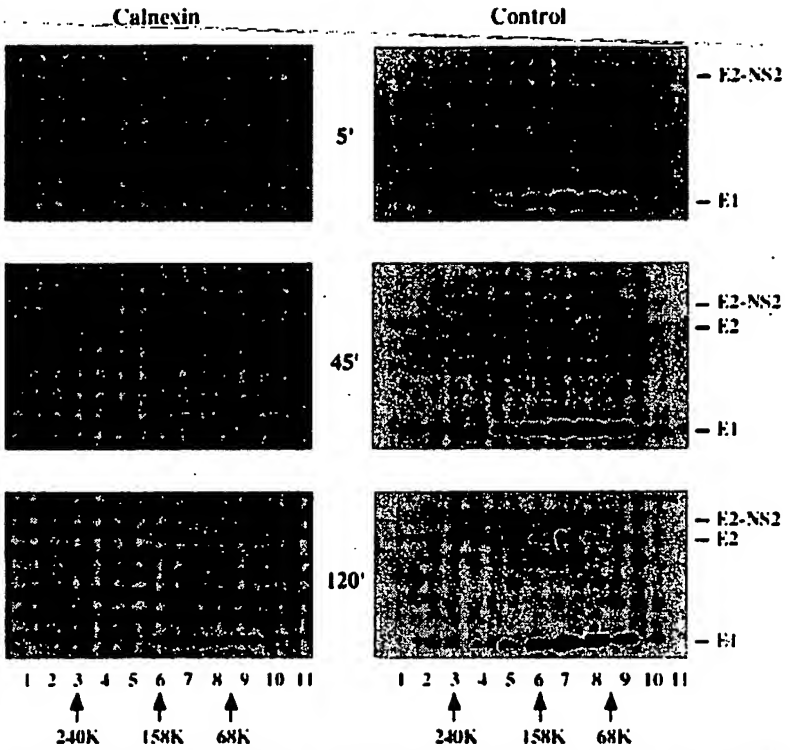

FIG. 9. Characterization of HCV glycoprotein oligomers associated with calnexin. Cells coinfected with vTF7-3 and vHCV1-1488 were pulse-labeled for 5 min and chased for the indicated times (in minutes). Labeled infected cells were lysed with 1% digitonin or 0.5% NP-40. Digitonin lysates were used for immunoprecipitation with anti-calnexin MAb, and coprecipitated HCV glycoproteins were dissociated from calnexin with NP-40. These released calnexin-associated complexes or total NP-40 extracts were sedimented through sucrose gradients (5 to 20%) as described in Materials and Methods. Fractions were collected and immunoprecipitated with anti-E1 MAb. The resulting samples were solubilized and separated by SDS-PAGE (10% polyacrylamide). HCV-specific proteins are indicated at the right. Molecular mass markers sedimented in a parallel sucrose gradient are indicated at the bottom.

duce a conformation which can be cleaved by host signal peptidase. Indeed, NS2 contains 5 Cys residues, and aberrant disulfide bridges between E2 and NS2 might be formed, inhibiting cleavage. However, since the kinetics of E2-NS2 processing were similar in the presence and absence of DTT, E2-NS2 cleavage does not appear to depend on a conformation which requires stabilization by disulfide bonds.

The individual HCV glycoproteins, as well as E1E2 complexes, coprecipitated with calnexin-specific MAb. This may indicate a direct association with calnexin, since mild conditions known to disrupt calnexin-glycoprotein complexes (NP-40 and heating at 37°C) dissociated HCV glycoproteins from these coprecipitates. The association of the HCV glycoproteins with calnexin was rapid, and their dissociation was slow, even after formation of E1E2 oligomers. Calnexin is a calcium-binding transmembrane protein found in the ER, and it associates with many newly synthesized, unfolded polypeptides (51). Upon proper folding, proteins typically dissociate from calnexin, leave the ER, and continue their journey through the secretory pathway. This has led to the suggestion that calnexin functions to prevent export of unfolded proteins and may also function as a molecular chaperone by assisting in folding and/or assembly. Recently, it has been shown that binding to calnexin is specific to molecules that possess monoglucosylated core glycans, suggesting that calnexin is a lectin-like protein (27, 31). However, calnexin can also bind nonglycosylated proteins (38), probably by interacting with their transmembrane domain (45). Strong evidence that calnexin functions to retain incompletely folded proteins in the ER comes from studies showing that the truncation of the cytoplasmic domain of calnexin does not interfere with its binding to substrate proteins but causes mislocalization of complexes within the cell (54).

In addition to facilitating folding and proper assembly of HCV glycoproteins, prolonged retention of HCV glycoprotein complexes in the ER may play a role in particle assembly. Our previous work showed that HCV glycoproteins localize predominantly to the ER network and do not migrate further than the *cis* Golgi (19). This suggests that some mechanism exists for their continued retention in this compartment and could reflect an important step in the assembly of HCV, if virions mature by budding through intracellular membranes, perhaps the ER, as has been proposed for some other viruses including flaviviruses (19, 52). In one model, E1E2-calnexin complexes might be retained in the ER until interaction with the budding nucleocapsid and formation of mature virions. Formation of these particles and dissociation from calnexin would allow the virions to begin movement through the secretory pathway and be released from infected cells. A similar model has been proposed for the assembly of other quaternary glycoprotein complexes (3). For instance, calnexin binds transiently to individual subunits of the major histocompatibility complex (class I and class II), the T-cell receptor, and the B-cell receptor prior to the assembly of mature ternary complexes, which then migrate to the plasma membrane (1, 14, 15, 35). Alternatively,

since E1E2 heterodimers can be released from calnexin yet still appear to remain in a pre-Golgi compartment (16), other signals for their intracellular retention may exist. In this case, dissociation of E1E2 complexes from calnexin may not be directly linked to virion assembly and secretion.

The slow folding of E1, as well as the presence of significant quantities of misfolded complexes, could reflect impaired function or suboptimal levels of critical ER chaperones under our expression conditions. Folding catalysts of the ER such as protein disulfide isomerase (PDI) accelerate the slow chemical steps, such as disulfide bond formation, that accompany protein folding. Recently, it has been shown that in vitro, the fate of reduced denatured lysozyme is dependent on whether it interacts first with heavy-chain-binding protein (BiP) or PDI (53). In addition, depending on the concentration of unfolded lysozyme relative to that of BiP and PDI, unfolded lysozyme either may be efficiently refolded into the native conformation or may form both soluble and insoluble complexes with BiP. The competition between the foldase/chaperone activity of PDI and the anti-chaperone activity of BiP has also been observed in vivo. During the expression of tissue plasminogen activator in Chinese hamster ovary cells (18), the level of tissue plasminogen activator secretion is inversely proportional to the level of BiP expression. Thus, an imbalance in either the ratio of PDI to BiP or the ratio of BiP to nascent chains may promote misfolding. Interaction between BiP and insoluble protein aggregates has been observed in vivo (6, 39, 46). Thus, aggregation, dependent on the anti-chaperone activities of BiP, PDI, and possibly other ER proteins, may represent a normal event during in vivo folding in the ER under conditions when the level of protein synthesis is above the chaperone capacity of the ER or when protein aggregation is kinetically faster than the association with chaperones.

Additional studies are needed to address the role of calnexin and other ER constituents in folding and assembly of HCV oligomers. It is hoped that by understanding the steps in this pathway, we can eventually express properly folded complexes at high levels to better study their functions in virus assembly, receptor binding, and entry. In addition, vaccination of chimpanzees with purified HCV glycoprotein complexes has been shown to confer partial protection against a low-dose homologous challenge (9). It is likely that the future success of this approach will depend, at least in part, on our ability to understand enough about HCV glycoprotein biogenesis to permit high-level production of properly folded immunogenic proteins.

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